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Trigonelline therapy confers neuroprotection by reduced glutathione mediated myeloperoxidase expression in animal model of ischemic stroke

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ABSTRACT

Aim: Stroke is devastating with a limited choice of intervention. Many pharmacological entities are available but none of them have evolved successfully in counteracting the multifaceted molecular alterations following stroke. Myeloperoxidase (MPO) has been reported to play an important role in neuroinflammation following neurodegenerative diseases. Therefore, using it as a therapeutic target may be a strategy to confer neuroprotection in stroke. Trigonelline (TG), a plant alkaloid has shown neuroprotective effects in the past. Here we explore its neuroprotective effects and its role in glutathione mediated MPO inhibition in ischemic stroke.

Methods: An in silico study was performed to confirm effective TG and MPO interaction. An in vitro evaluation of toxicity with biochemical estimations was performed. Further, in vivo studies were undertaken where rats were treated with 25, 50 and 100 mg/kg TG or standard MPO inhibiting drug4-Aminobenzoic hydrazide (4-ABH) at 60 min prior, post immediate and an hour post 90 min of middle cerebral artery occlusion (MCAo) followed by 24 h reperfusion. Rats were evaluated for neurodeficit and motor function tests. Brains were further harvested for infarct size evaluation, biochemical analysis, and western blot experiments.

Key findings: TG at 100 mg/kg dose i.p. administered immediately post ischemia confers neuroprotection by reducing cerebral infarct with improvement in motor and neurodeficit scores. Furthermore, elevated nitrite and MDA levels were also found to be reduced in brain regions in the treated group. TG also potentiated intrinsic antioxidant status and markedly inhibited reduced glutathione mediated myeloperoxidase expression in the cortical brain region.

Significance: TG confers neuroprotection by reduced glutathione mediated myeloperoxidase inhibition in ischemic stroke.

1. Introduction

Neuroinflammation is a major threat following stroke with limited option for therapeutic intervention [1]. Aberrant neuroinflammatory response is often noxious to neurons that may lead to exacerbation of stroke pathology [2]. Alteration to the biochemical milieu is detrimental while generation of free radicals also poses challenges for its treatment [2,3].

Increased oxidative stress burden, compromised antioxidant system, overproduction and release of pro-inflammatory mediators and enhanced expression of myeloperoxidase (MPO) are prominent and subsequent to stroke [4,5]. Ischemic stroke leads to activation of cytokines, microglia, leukocytes and other inflammatory markers leading to generation of reactive oxygen species (ROS) that contribute to the production of hydrogen peroxide (H_2O_2) which is converted to hypochlorous acid (HOCl) in the presence of MPO protein [3,6]. MPO is reported to increase following neurodegenerative diseases and is associated with cellular damage which can lead to neuronal insult [7,8].

Abbreviations: MDA, Malondialdehyde; NO, Nitric Oxide; TTC, 2,3,5-triphenyltetrazoliumchloride; MPO, Myeloperoxidase; HOCl, Hypochlorous acid; pdb, Protein Data Bank; MCAo, Middle Cerebral Artery Occlusion

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HOCl diffuses from the plasma membrane and inhibits the intracellular enzymes from further interacting with adenosine triphosphate (ATP) to alter the energy metabolism process [9]. It also inhibits mitochondrial respiration by decreasing intracellular nicotinamide adenine dinucleotide (NAD), ATP and glutathione (GSH) [10]. In brain pathology, there is a continuous release of HOCl from peripheral leukocytes and microglia which further diffuse to the brain parenchyma causing a serious threat to the brain by altering its molecular targets [11].

Use of nutraceuticals targeting neuroinflammation has been suggested as a novel therapeutic approach for neurodegenerative and neuroinflammatory disorders [12,13]. Trigonelline (TG), a plant alkaloid obtained from fenugreek (*Trigonella foenum-graecum* L.) seeds is reported to have medicinal benefits and has shown several pharmacological activities including acetylcholinesterase inhibitory effects, anti-inflammatory, anti-apoptotic, anti-hyperglycemic, anti-hyperlipidemic and neuroprotective activities with cognition improvement potentials [14–17]. TG is also one of the major components found in coffee, present in similar amounts than those of caffeine [18]. TG is a product of niacin (vitamin B3) metabolism and has shown to lower lipid peroxidation and potentiate antioxidant defensive system with a promising role in regenerating dendrites and axons to improve cognitive functions [19,20]. Owing to the mentioned spectrum of neuroprotective properties of TG, in the present study we have looked into its neuroprotective spectrum in rodent model of brain ischemia/reperfusion with a relation to reduced GSH mediated MPO inhibition.

2. Materials and methods

2.1. Chemicals

TG, triphenyl tetrazolium chloride (TTC) and other chemicals were obtained from Sigma-Aldrich and Abcam unless otherwise specified. All the chemicals and enzymes were used as per manufacturer's instructions.

2.2. Ethics statement

The study was approved by the Institutional Animal Ethical Committee of the Animal House of NIPER-A (Approval No-NIPERA/IAEC/2017/008).

2.3. In silico studies

2.3.1. Molecular docking studies

Molecular modelling studies were done by using GLIDE (Grid-based Ligand Docking with Energetics) module of Schrodinger software running on Linux 5 (RHEL5) workstation. Maestro Graphical User Interface (GUI) workspace was used for all four steps involved in docking: ligand preparation, protein preparation, grid generation and Extra Precision docking (XP-mode). Initially, the crystal structure of MPO (PDB ID: 5FIW) was obtained from a RCSB protein data bank (PDB) using “Prep Wizard” module of Schrodinger suite. After importing the protein, ‘protein preparation’ was done by adding hydrogen and removing water molecules beyond 5 Å of the binding pocket. A grid was generated using “Glide Grid generation” system using the default options for docking studies. The structures of the synthesized compounds were drawn in Schrodinger and refined using “Ligprep” module. Every single docking calculation was performed using the XP mode of GLIDE program. The results of the ligand docking are expressed as Docking score or Glide score (preferably docking score) which signify the strength of the non-covalent interaction between the ligand and the protein. Glide score is an empirical scoring function that approximates the ligand binding free energy. It is primarily concerned with generating an accurate pose for each protein-ligand complex and separating ligands with appreciable binding affinity from those that don't bind, in a ranked list. Docking score is usually used to assess the docking of ligands. The more negative value of these scores, the more stable is the ligand-protein complex formed, thus greater is the binding affinity. The docking score and the Glide Score are the same unless Epik state penalties are included in the scoring (the recommended and default behaviour). The molecules were also observed for docking score and appropriate docking pose in the cavity [21].

2.4. In-vitro studies

2.4.1. Cell culture and maintenance of cell line

The rat adrenal medulla PC12 (pheochromocytoma) cell line was purchased from National Centre for Cell Sciences (NCCS), Pune, India. These cells were grown and maintained using DMEM-HG medium with 1% Penicillin/streptomycin and 10% fetal bovine (FBS) and placed in an incubator (Thermo Fisher) with 5% CO_2 and 95% air at 37 °C. When confluent, all the cells were trypsinized using trypsin-EDTA (HiMedia, Mumbai) and the cells were seeded in culture plates.

2.4.2. MTT assay

The cytotoxicity produced by molecule of interest is the preliminary step for evaluation to estimate its toxic dose. MTT is taken by cells which are alive and gets reduced to formazan crystals, which is a purple coloured water insoluble product. The ability of cells to reduce 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT), reflects the mitochondrial integrity and its activity, which in turn may be interpreted as a measurement of viability [22]. 5000 cells/well were seeded in 96 well plate and incubated with 200 µl media for 24 h. After 24 h, media was decanted and fresh media was added to the wells. 3 µM sodium azide (NaN_3) was added to induce hypoxia and the plate was incubated for 4 h after which media was decanted and fresh media was added. The cells were then treated with TG for 4 h at different concentrations of 25, 50, 100 and 200 µM. 20 µl MTT (5 mg/ml) was then added to each well and plate was incubated for 4 h. 100 µl dimethyl sulfoxide (DMSO) was added to each well for solubilizing the purple coloured formazan crystals and absorbance was measured at 570 nm using a micro plate reader (Multiscan Go; Thermo Fisher) [23].

Calculation:

$$\%\ \text{Viability} = \frac{\text{Mean Absorbance of sample}}{\text{Mean Absorbance of control}} \times 100$$

2.4.3. Preparation of cell lysate

10^5 cells/well were seeded in a 6 well plate and incubated till 80% confluency was attained. Media was decanted and fresh media was added to the wells. 3 µM $NaN_3$was added and plate was incubated for 4 h after which media was decanted and fresh media was added. The cells were then treated with TG for 4 h at different concentrations of 25, 50 and 100 µM. Media was then removed and cells were washed twice with phosphate buffer saline (PBS). Cells were scraped in 500 µl lysis buffer and collected in a microcentrifuge tube. Cells were sonicated and then centrifuged at 12,000*g* for 20 min. The supernatant obtained was collected and used for the estimations. Protein concentration was determined using bicinchoninic acid (BCA) reagent.

2.4.4. Biochemical analysis

To perform biochemical analysis we measured nitrite, reduced GSH and malondialdehyde (MDA). GSH is an important antioxidant present in cells which detoxifies xenobiotics via conjugation, thereby protecting protein integrity and function [24]. GSH is also an intracellular redox buffer protecting the cell from oxidative damage. Under oxidative stress, H_2O_2 is reduced in the presence of glutathione peroxidase (GPx) to H_2O and at the same time reduced GSH is oxidised to GSSH [25]. This mechanism helps the cell from oxidative damage [26]. Following hypoxia, there is a rapid increase in the levels of intracellular calcium, which activates neuronal nitric oxide synthase (nNOS) in the presence of protein kinase C and calmodulin dependent kinase (CDK). This nNOS

activation leads to the generation of nitric oxide (NO). NO at high concentrations can damage the neuronal cells by forming peroxynitrite anion after combining with superoxide anion [4]. We performed assay to assess the level of MDA which is a marker for polyunsaturated fatty acid peroxidation in cells. Lipid peroxidation gives a measure of cellular membrane damage which in turn is related to an increase in free radical production [27]. For analysis of GSH levels, 100 µl of sample was mixed with 100 µl of Ellman's reagent in 0.1 M phosphate buffer (pH 8.0). The mixture was then incubated for 10 min at 38 °C in a water bath. Absorbance was measured at 412 nm using a micro plate reader [28]. For nitrite estimation, 100 µl of sample was added in a 96 well plate, to which 100 µl of working Griess reagent and 50 µl of water was added and incubated for 30 min at room temperature. Absorbance was measured at 540 nm [29]. MDA was estimated by taking 100 µl sample, 100 µl sodium dodecyl sulphate (SDS), 750 µl thiobarbituric acid (TBA), 300 µl water and 750 µl acetic acid were taken in a 2 ml centrifuge tube. The above mixture was placed in water bath for 1 h at 95 °C after which 250 µl of the mixture was added to a 96 well plate and absorbance was taken at 532 nm [4]. GSH, nitrite and MDA concentrations were calculated using standard curves and the amount obtained was normalised with total protein content of each well.

2.5. In-vivo studies

2.5.1. Animals

Adult (6–7 weeks) Sprague–Dawley male rats weighing 240–270 g were used. All animals were quarantined for 6 days and were maintained in controlled environmental conditions like 10% air exhaust air conditioning unit, temperature of 25 ± 0.5 °C and relative humidity of 60 ± 5%, 12 h of light and dark cycle. Water was provided ad libitum to animals during experimental period. Investigators were blinded to treatment group allocation during surgery and during outcome evaluations.

2.5.2. Animal surgery

Male rats were exposed to reversible middle cerebral artery occlusion (MCAo) following an overnight fast. 3% isoflurane and 70% nitrous oxide were used to induce anaesthesia. Catheterization of the right femoral artery and vein were performed for monitoring of blood pressure and arterial blood sample collection with blood gas and glucose assessments. Vecuronium bromide of 0.35 mg/kg i.v. was administered for immobilization of rats. Endotracheal intubation and mechanical ventilation were done with a mixture of 0.5–1% isoflurane, 70% nitrous oxide with a balance of oxygen. Ventilator (VentElite, Harvard Apparatus) was used for arterial PCO_2 and PO_2 maintenance. Rectal temperature was maintained at 37–37.5 °C by heating lamps and was measured continuously. Cranial temperature was separately monitored by a 29-gauge thermocouple implanted into the right temporalis muscle and was maintained at 36–36.5 °C by a warming lamp placed above the rat's head throughout the experiment [30,31]. Our previous studies have shown that the cranial temperature 36–36.5 °C corresponds to a brain temperature of 36.5–37 °C [30]. The previously well-described filament technique for MCAo was performed in this study [32–35]. For the sham group, similar surgical procedures where performed, however, the filament insertion step was omitted.

2.5.3. Cerebral blood flow (CBF) monitoring

CBF was monitored continuously throughout the experiment by laser Doppler flowmetry (LDF, AD Instruments) as per reported methodology [31]. The left scalp was opened, and the skull was exposed with a 2-mm burr hole drilled on the left sphenoid bone (0.5 mm anterior; 6 mm lateral to bregma). The dura was kept intact. The Doppler probe was placed above the dura and the blood flow through the cortical branch of the MCA was monitored. CBF was measured in terms of perfusion units (PFU). The LDF signals were recorded prior to, during and after the suture insertion. Rats not exhibiting 70% reduction in cerebral blood flow were excluded from the study.

2.5.4. Reversible middle cerebral artery occlusion (MCAo)

The surgical procedures were performed as per previous reported methodology [31]. The left common carotid artery (CCA) was exposed through a midline incision on the neck and separated from the surrounding tissue including the vagus nerve by blunt dissection using microsurgery. Left external carotid artery (ECA), were dissected and coagulated under an operating microscope. Two long 5–0 silk sutures were tied to the ECA as distal to the carotid bifurcation as possible with transient CCA and internal carotid artery (ICA) clamping. Two short (3 cm) 5–0 silk sutures were tied loosely around the segment of ECA. The left ICA was exposed, and the origin of the pterygopalatine artery (PPA) visualized. An intraluminal 3–0 silicon coated suture, was introduced into the distal segment of ECA via a small incision on the vessel and advanced into the ECA lumen to reach the bifurcation. The ECA was distally cut between the 2 long-suture nodes. The 3–0 suture was then turned into the ICA. The suture was advanced into the distal ICA until feeling resistance to hit the bifurcation of MCA and confirming a sharp drop of regional cerebral blood flow by the LDF. A sharp decrease in the LDF signal was interpreted to indicate a successful MCA occlusion. Withdrawal of suture was done after the 90 min of occlusion period. During this procedure and recirculation period, blood pressure, blood gases, blood glucose, cranial and rectal temperatures were maintained (Table 1). Sham operated rats underwent the same surgical procedure, except MCAo.

2.5.5. Dose optimization and experimental design

TG dissolved in normal saline was administered intraperitoneally at three time points, 30 min before MCAo, immediately after MCAo and 1 h post MCAo. TG was administered in three doses of 25, 50 and 100 mg/kg to determine the optimum neuroprotective dose. 24 h after TG treatment, animals were sacrificed and brains were harvested for assessing infarct volume, biochemical estimations and western blotting (n = 6 was used for each experimental group). 4-Aminibenzoic hydrazide (4-ABH), a specific and irreversible inhibitor of MPO was used as a standard drug for comparison. Pictorial representation of the experimental design is provided in Fig. 1.

2.5.6. Neurodeficit scoring and motor deficit test

Neurobehavioral battery test was conducted as previously described [31]. A range from normal score of 0 to 12 was taken, which included tests for proprioception, postural reflex and sensorimotor integration. For motor function test, we performed the rotarod motor test. The rats were trained for 3 consecutive days before MCAo. The rats were placed

Table 1
Table representing the general physiological parameters observed during experiment.

Parameters studied	Pre-ischemia	Ischemia (60 min)	Ischemia (90 min)	Post ischemia (120 min)
pH	7.42 ± 0.003	7.299 ± 0.002	7.257 ± 0.001	7.390 ± 0.001
pO_2 (mm Hg)	119 ± 2	116 ± 3	107 ± 2	115 ± 2
pCO_2(mm Hg)	44.7 ± 2	47 ± 2	50 ± 2	51 ± 2
Glucose (mmol/l)	6.3 ± 0.6	6.6 ± 0.4	6.2 ± 0.6	6.6 ± 0.3
HCO_3^- (mmol/l)	24.43 ± 2.33	23.92 ± 1.28	24.01 ± 1.94	24.21 ± 2.10

Rectal temperature was maintained at 37 ± 0.5 °C and brain temperature was maintained at 36 ± 0.5 °C.

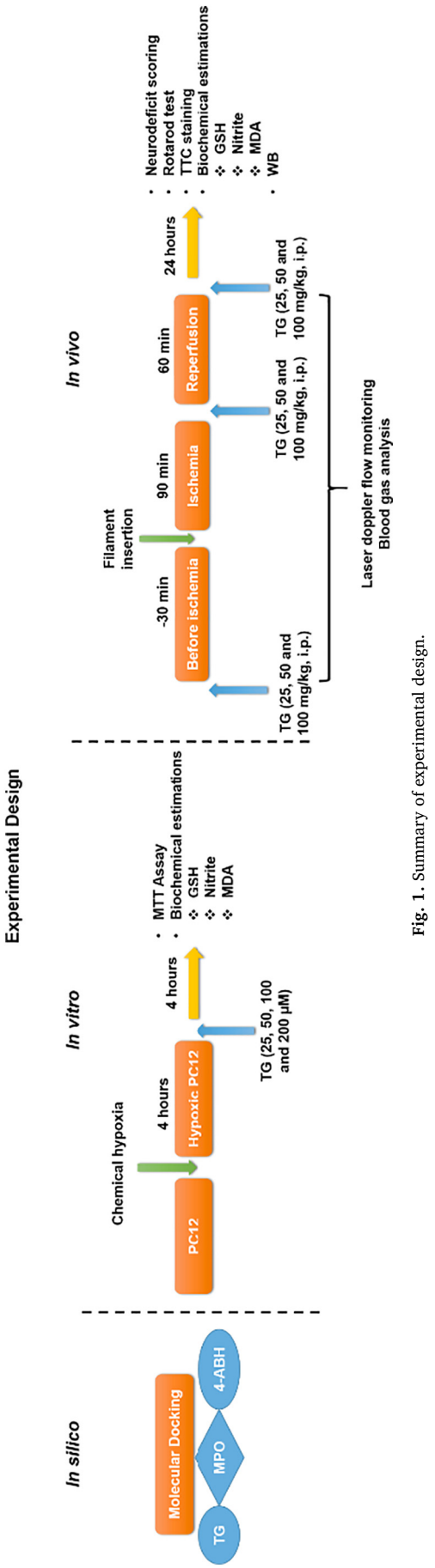

Fig. 1. Summary of experimental design.

on the rotarod cylinder and latency to fall was measured. The speed was gradually increased from 10 to 30 revolutions per minute (rpm) over 5 min. The trial was halted if the rat fell off the device and if it spun passively for 2 consecutive revolutions without attempting to walk. The mean duration (in seconds) was recorded from 3 rotarod measurements 1 day before surgery. The rats were again tested at 24 h after MCAo.

2.5.7. Brain infarct analysis

After neurological examination rat brains were harvested on ice. Coronal sections 2 mm thick were taken from the region beginning 1 mm from the frontal pole and ending just rostral to the cortico cerebellar junction in a brain matrix and were transferred at −20 °C. The slices were incubated in TTC (0.5% in 0.1 M PBS) at 37 °C for 30 min. TTC stains viable brain tissue brick red whereas unstained brain tissue i.e. infarcted portion of tissue remains unstained and appears as white. The volume of infarct was measured and quantified by image analysis software (NIH image J) [4].

2.5.8. Tissue lysate preparation and biochemical analysis

Brains were isolated on ice. 75–100 mg of cortex was added to 1 ml of lysis buffer with protease inhibitors. The tissue was triturated with a pestle. The triturated tissue was then sonicated (8 on 8 off cycle, 40 amplitude) three times and then centrifuged at 12000*g* at 4 °C for 20 min. The supernatant was collected for various biochemical assays and western blotting techniques. GSH, nitrite and MDA levels were analyzed in a similar manner as mentioned in the in vitro studies. GSH, nitrite and MDA concentrations were calculated using standard curves and the amount obtained was normalised with total protein content of each well.

2.5.9. Western blot analysis

For western blotting, 30 μg of tissue lysate was denatured in gel loading buffer (100 mM Tris–Cl (pH 6.8), 2% SDS, 20% glycerol and 0.2%bromophenol blue) in dry bath at 95 °C for 5 min. The samples were loaded on 10% SDS–polyacrylamide gel along with ladder. Electrophoresis was carried out in gel running buffer containing 250 mM glycine, 25 mM Tris, and 0.1% SDS. After electrophoresis, proteins were transferred onto the PVDF membrane in trans blotting system (Bio-Rad) for 45 min with constant power supply of 80 V. After transfer, the membrane was blocked in 3% BSA for 2 h. The blot was then incubated with primary antibodies of MPO (1 μg/ml; abcam, ab45977) and GAPDH (1:5000; abcam, ab9485) overnight at 4 °C. After three washes with TBST (TBS (tris buffered saline) + 0.05% Tween-20) for 5 min each, the blot was incubated with HRP-conjugated goat anti-rabbit secondary anti-body (abcam, ab6721). After washing with TBST, proteins were revealed with electrochemiluminescence (ECL) (Invitrogen) and their expression level was measured by densitometry. GAPDH was used as control for immune-blotting. Band density values were normalised to GAPDH.

2.5.10. Statistical analysis

The data is presented as mean value ± SEM. A two way analysis of variance (ANOVA) followed by a multiple comparison procedure (Bonferroni post hoc test) was used for statistical differences among groups. In all cases, P value < 0.05 was considered statistically significant.

3. Results

3.1. In silico studies

TG interacts with the protein via formation of a hydrogen bond with *N*-accetylglucosamine (NAG), arginine (ARG) and tryptophan (TRP). From Table 2 we can say that glide score and docking score of TG (−4.036 and −4.036 respectively) is better as compared to 4ABH (−3.683 and −3.683 respectively) (Fig. 2; Table 2). This suggests that

Table 2
Docking scores of trigonelline (TG) and 4-Aminobenzoic hydrazide (4-ABH) with myeloperoxidase (MPO).

S. no.	Ligand	Glide score	Docking score	Epik score
1.	Trigonelline	−4.036	−4.036	0
2.	4-Aminobenzoic hydrazide	−3.683	−3.683	0

TG may have better binding affinity to MPO than the standard MPO inhibitor 4ABH.

3.2. In vitro studies

3.2.1. Effect of TG on cell viability

Cell viability was measured using MTT assay method. 25, 50 and 100 μM of TG concentration showed an increase in the cell viability following hypoxia induction, with 100 μM concentration showing an increase (***P < 0.05). However, 200 μM TG concentration did not show any increase in cell viability following induction of hypoxia. For further studies 25, 50 and 100 μM concentrations of TG were used (Fig. 3).

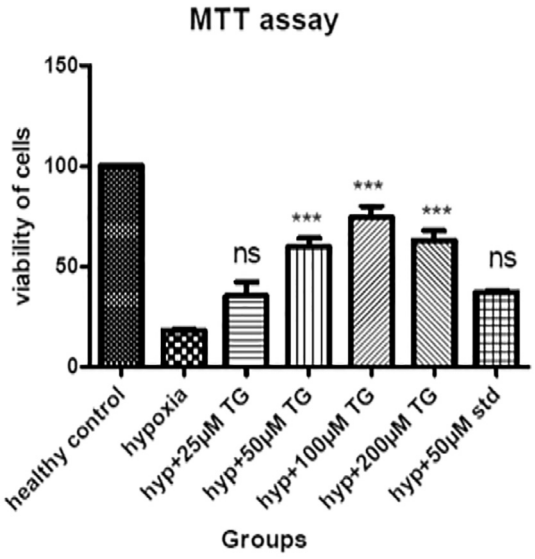

Fig. 3. Effect of TG on viability of PC12 cells. The data is expressed as mean ± SEM and analyzed for statistical significance using two way ANOVA with Bonferroni post hoc test (***P < 0.05 vs hypoxia group).

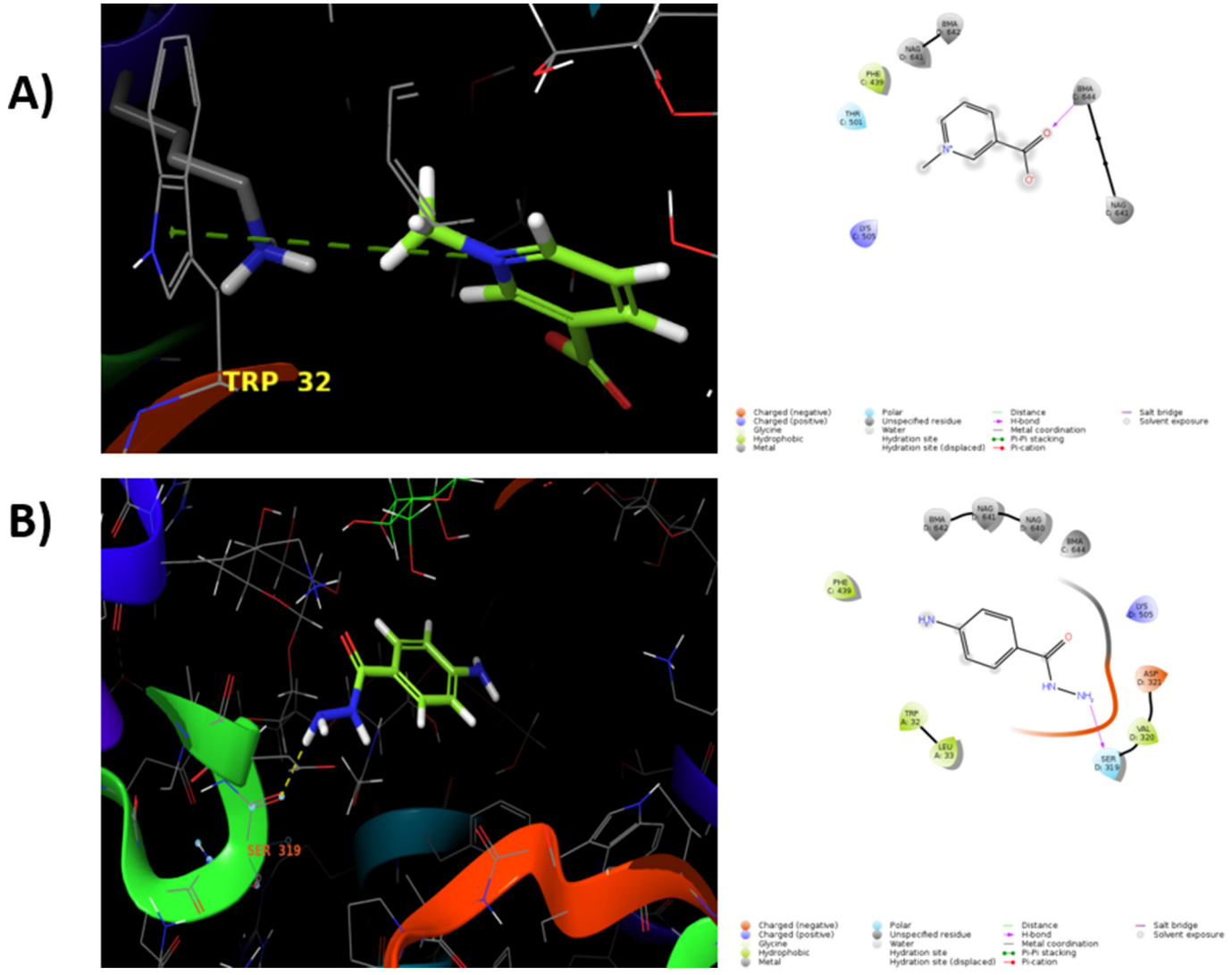

Fig. 2. In-silico molecular docking studies; (A) Interaction of TG with MPO PDB ID (5FIW), (B) Interaction of 4-ABH with MPO PDB ID (5FIW), 3D and 2D representation.

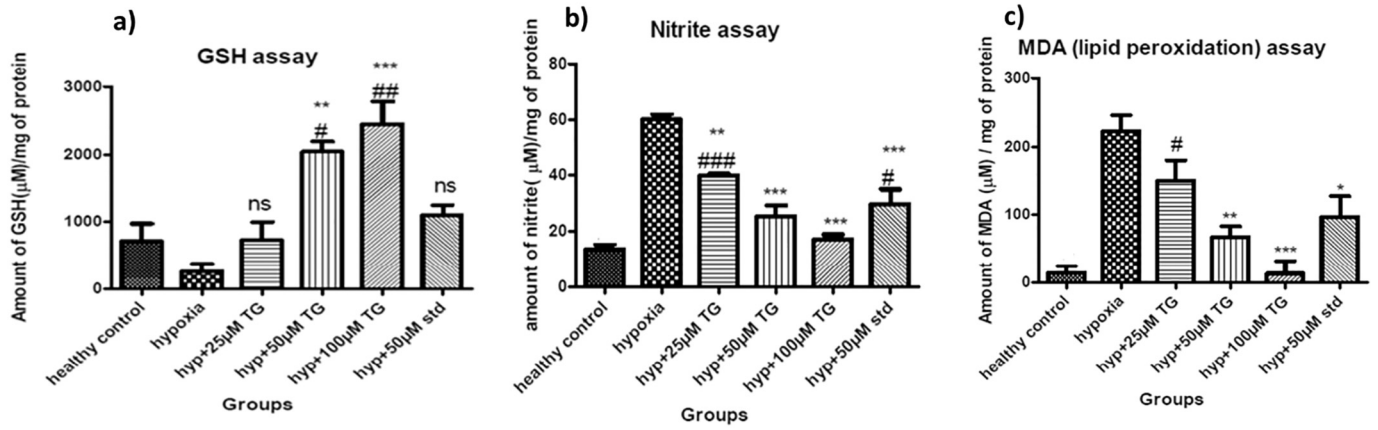

Fig. 4. a) Graph representing changes in in vitro GSH levels following treatment of PC12 cells with TG. The data is expressed as mean ± SEM and analyzed for statistical significance using two way ANOVA with Bonferroni post hoc test (***P < 0.001; **P < 0.01; $^{\#\#}$P < 0.01; $^{\#}$P < 0.05; $^{\#}$vs healthy control group; *vs hypoxia group). b) Graph representing changes in in vitro nitrite levels following treatment of PC12 cells with TG. The data is expressed as mean ± SEM and analyzed for statistical significance using two way ANOVA with Bonferroni post hoc test (***P < 0.001; **P < 0.01; $^{\#\#\#}$P < 0.001; $^{\#}$P < 0.05; $^{\#}$vs healthy control group; *vs hypoxia group). c) Graph representing changes in in vitro MDA levels following treatment of PC12 cells with TG. The data is expressed as mean ± SEM and analyzed for statistical significance using two way ANOVA with Bonferroni post hoc test (***P < 0.001; **P < 0.01; *P < 0.5; $^{\#}$P < 0.05; $^{\#}$vs healthy control group; *vs hypoxia group).

3.2.2. Effect of TG on GSH, nitrite and MDA levels

GSH levels were decreased (Fig. 4) while nitrite (Fig. 5) and MDA (Fig. 6) levels were increased (*P < 0.05) in the hypoxia group as compared to healthy control. TG at concentrations of 50 and 100 μM showed an increase in GSH levels and the difference was found to be significant (*P < 0.05) as compared to the hypoxia group where 3 μM NaN_3 was used to induce hypoxia. TG at concentrations of 25, 50 and 100 μM showed a dose dependent (*P < 0.05) decrease in nitrite and MDA levels as compared to the hypoxic group. TG at concentrations of 50 and 100 μM showed (*P < 0.05) increase in GSH levels as compared to 4-ABH while all three concentrations of TG showed (*P < 0.05) reduction in nitrite and MDA levels as compared to 4-ABH.

3.3. In vivo studies

3.3.1. Dose optimization of TG

The minimum effective neuroprotective dose of TG was determined on the basis of neurological deficit reduction and decrease in the volume of brain infarct in rats subjected to 90 min ischemia and 24 h of reperfusion injury. In our study, the ischemia-reperfusion injury produced consistently marked infarctions in both cortical and subcortical ipsilateral areas of the rat brains as confirmed by the TTC stained brain coronal sections (Fig. 5b and c). When treated with different doses of TG at different time points, the 100 mg/kg dose when given prophylactically or immediately following stroke, showed a significant reduction in the infarct area as compared to the stroke group.

On the basis of neurological scores obtained following ischemic-reperfusion injury, neurological deficits in rats were analyzed (Fig. 5d). The rats in the stroke group displayed a higher score as compared to the sham group while improvement was seen in neurological deficit score of the animals treated with different doses of TG. The group in which TG 100 mg/kg was given immediately following stroke showed a significant level of reduction in the neurodeficit score as compared to the stroke and other treatment groups. Therefore, 100 mg/kg of TG given immediately following stroke was selected as the optimum dose.

3.3.2. Effect of TG on motor function

A significant decrease in retention time on the rotating rod was observed in the stroke group as compared to the sham and healthy groups at 10, 20 and 30 rpm. TG treatment was able to significantly increase the retention time on the rod (Table 3).

3.3.3. Effect of TG on GSH, nitrite and MDA levels

We found that there is a significant increase in GSH levels (Fig. 6a) in the group treated with TG 100 mg/kg given immediately following stroke as compared to the stroke group. Also nitrite (Fig. 6b) and MDA (Fig. 6c) levels were significantly reduced in the same group as compared to the stroke group. The levels resembled pre-stroke levels. TG fared better in normalizing the levels of GSH, nitrite and MDA in comparison to the 4-ABH group. However, the difference was not significant.

3.3.4. Effect of TG on MPO expression

MPO expression was found to be significantly higher in the stroke group as compared to the sham group. MPO expression was seen to be significantly decreased in the TG 100 mg/kg treated group as compared to the stroke group. Also TG was also found to be superior when compared to 4-ABH in lowering the levels of MPO (Fig. 7).

4. Discussion

Natural entities have piqued interest for their neuroprotective roles in brain pathology. In the present study, TG, a plant alkaloid has shown neuroprotection in a dose-dependent manner in rodent model of ischemic stroke. Prior studies have highlighted the neuroprotective role of TG against various neurological disorders [36–38]. Hence, we looked further into the probable involvement of MPO in rendering neuroprotection and also the plausible involvement of reduced GSH as one of its mediators. The objective of the study was to identify the effects of TG administered post ischemia in inhibiting formation of reduced GSH mediated MPO.

Molecular docking studies were performed to confirm the interaction of TG with MPO. The docking scores of MPO were compared to that of 4-ABH that reported to inhibit the formation of HOCl by irreversibly inactivating the MPO enzyme [39]. TG interacted with MPO by forming hydrogen bond with NAG, ARG, and TRP. The glide scores and docking scores of TG were found to be better than the glide score and docking score of 4-ABH (Fig. 2). The better docking and glide scores of TG and 4-ABH suggests TG to be more potent inhibitor of the MPO enzyme than 4-ABH.

In vitro studies were performed to study the effect of TG on PC12 cell lines. Cell viability assay for cytotoxicity of TG showed that the viability of cells was significantly reduced at 200 μM as compared to that of control group. However, TG at 25, 50 and 100 μM

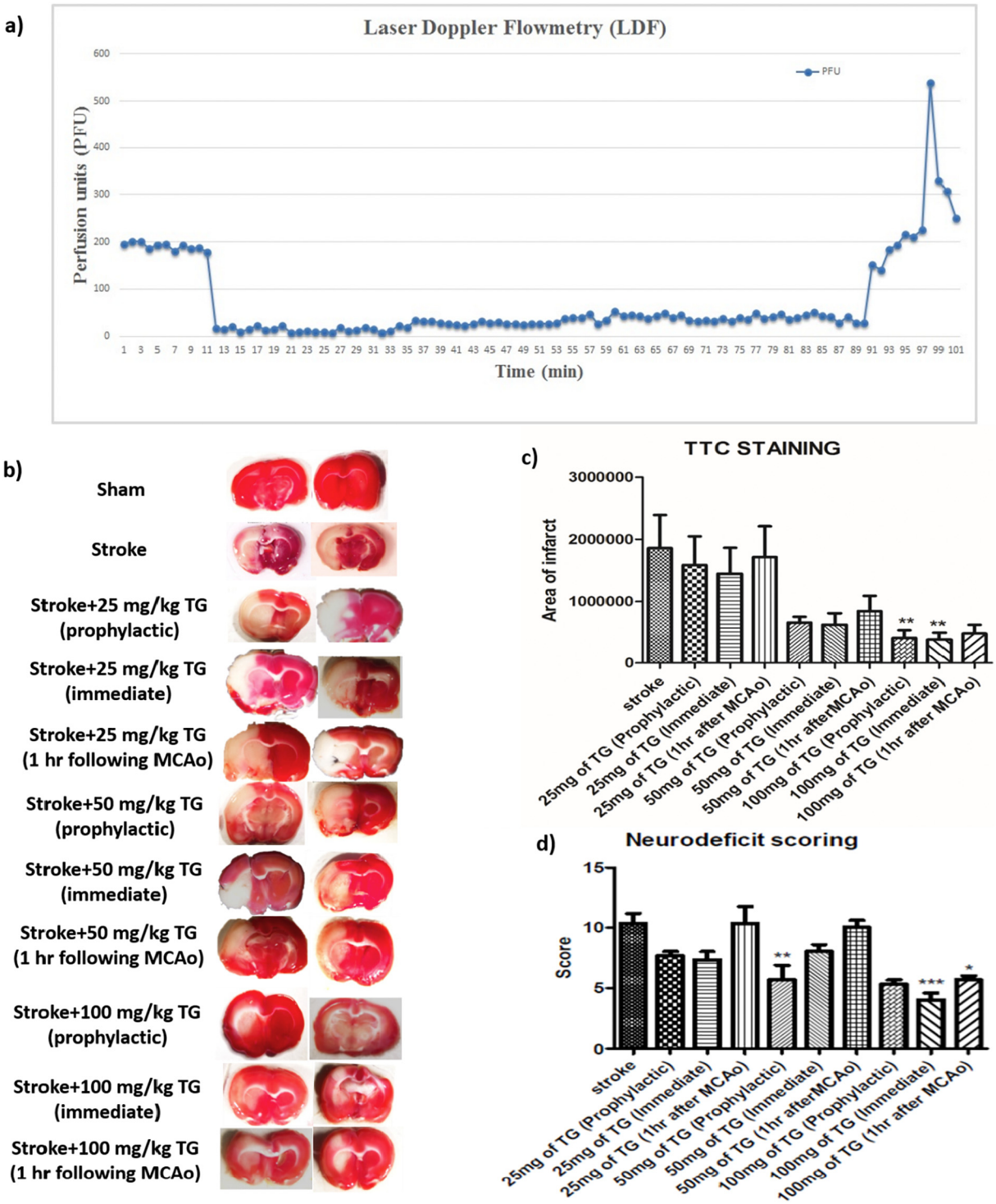

Fig. 5. a) Image depicting the changes in the cerebral blood flow during MCAo induction as measured by Laser Doppler Flowmetry. b) TTC stained coronal section of rat brain showing area of infarct. c) Graph representing comparative area of brain infarct between different treatment groups. The data is expressed as mean ± SEM and analyzed for statistical significance using two way ANOVA with Bonferroni post hoc test (**P < 0.01; *vs stroke group). d) Graph representing comparative neurodeficit score between different treatment groups. The data is expressed as mean ± SEM and analyzed for statistical significance using two way ANOVA with Bonferroni post hoc test (**P < 0.01, ***P < 0.1; *vs stroke group).

concentrations showed a concentration dependent increase in cell viability. When compared to the hypoxia group, TG at 50 and 100 μM concentrations showed significant increase in cell viability while at 25 μM TG did not show any significant increase in cell viability.

Ischemia/reperfusion injury exacerbates free radical production causing undiscerning damage of biological macromolecules including membrane lipids, proteins, and nucleic acid. Free radical scavenging effect of TG may be one of the prime mechanism of neuroprotection against ischemic injury [19,40,41]. Hence, TG may evolve as a molecule of choice with other therapies to prevent neuronal injuries associated with ischemic stroke. Our study reveals an increase in nitrite levels following NaN_3 treatment. The levels appeared similar to the

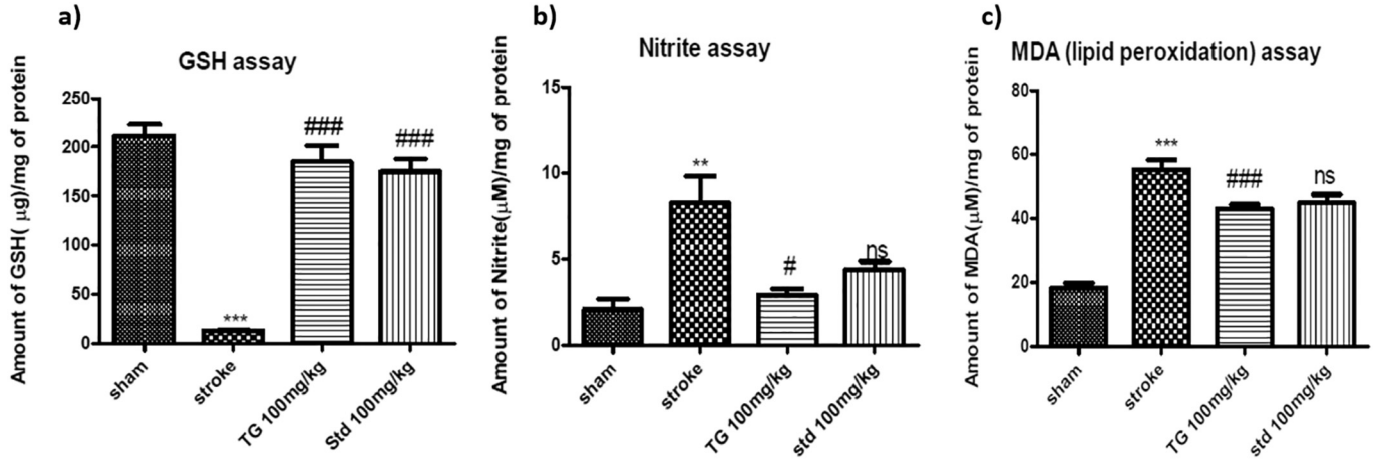

Fig. 6. a) Graph representing changes in in vivo GSH levels following treatment of rats with TG. The data is expressed as mean ± SEM and analyzed for statistical significance using two way ANOVA with Bonferroni post hoc test (***P < 0.001; $^{###}$P < 0.001; *vs sham group; #vs stroke group). b) Graph representing changes in in vivo nitrite levels following treatment of rats with TG. The data is expressed as mean ± SEM and analyzed for statistical significance using two way ANOVA with Bonferroni post hoc test (**P < 0.01; $^{#}$P < 0.05; *vs sham group; $^{#}$ vs stroke group; ns vs stroke group). c) Graph representing changes in in vivo MDA levels following treatment of rats with TG. The data is expressed as mean ± SEM and analyzed for statistical significance using two way ANOVA with Bonferroni post hoc test (***P < 0.001; $^{###}$P < 0.001; *vs sham group; $^{#}$ vs stroke group; ns vs stroke group).

Table 3
Table representing time spent on rotarod of control and experimental rats. Values expressed as Mean ± SEM.

Groups	Mean time (sec)		
	10 Rpm	20 Rpm	30 Rpm
Healthy control	180	177.33 ± 2.66	180
Sham group	180	180	180
Stroke group	15 ± 2.48	16.86 ± 6.66	5.3 ± 2.81
TG (25 mg/kg prophylactic)	9 ± 0.57	6 ± 0.57	2.67 ± 0.33
TG (25 mg/kg immediate)	5.22 ± 0.48	8.03 ± 0.21	8.95 ± 0.81
TG (25 mg/kg 1 h after MCAo)	3.01 ± 0.11	3.83 ± 0.14	5.51 ± 0.31
TG (50 mg/kg prophylactic)	4.78 ± 0.25	3.18 ± 0.20	2.2 ± 0.17
TG (50 mg/kg immediate)	5.59 ± 0.22	4.05 ± 0.07	3.03 ± 0.08
TG (50 mg/kg 1 h after MCAo)	9.51 ± 0.86	10.83 ± 0.16	4.12 ± 0.56
TG (100 mg/kg prophylactic)	13.51 ± 0.36	12.07 ± 1.00	9.83 ± 1.14
TG (100 mg/kg immediate)	24.44 ± 0.76**	20.52 ± 0.92*	16.57 ± 0.93*
TG (100 mg/kg 1 h after MCAo)	18.16 ± 1.27	12.36 ± 0.68	9.04 ± 0.21

** P < 0.01.
* P < 0.05; *vs stroke group.

sham group following treatment with TG in a concentration dependent manner and TG was more effective in comparison to 4-ABH in normalizing the nitrite levels. Further, we looked into the status of lipid peroxidation, which is evident from elevated levels of MDA within the ischemic brain region immediately to post reperfusion [42–44]. We found that TG was able to reduce MDA in a concentration dependent manner.

A hypoxic episode reduces the GSH concentration in the cell. This occurs due to reduced cystine uptake and a concomitant decrease in GSH [26]. In our study we found that TG was able to increase the reduced GSH levels in hypoxic PC12 cells.

With promising results obtained from in vitro studies, we proceeded to study the neuroprotective role of TG in rat model of ischemic stroke. In vivo dose optimization of TG were done on the basis of neurodeficit scoring and TTC [45]. As ischemic stroke involves cognitive, behavioural and locomotor impairments [46–49], results showed that rats those were administered with 100 mg/kg TG immediately following ischemia had significant reduction in brain infarct volume as well as improved neurodeficit scores. The optimized dose was further used for in vivo studies for different parameters.

Rotarod test was performed on rats to observe the effect of TG on motor incoordination. Rats in the stroke group demonstrated a lower retention time than the sham group while the groups treated with TG demonstrated a higher retention time.

Interaction between brain parenchymal cells, endothelial cells, and the antioxidant enzyme system in capillary endothelial cells post ischemia often plays a crucial role in governing the degree of hypoxic/ischemic brain injury. It leads to a persistent reduction in the activities of enzymes such as GPx, and GSH reductase, signifying the increase in the vulnerability of BBB to oxidative damage during reoxygenation [50]. GSH levels were found to be significantly reduced in the stroke group. The levels were found to be increased following treatment with optimized dose of TG following stroke. Hence, it may be suggested that TG in the optimized dose was able to potentiate the intrinsic antioxidant defence system.

As previously reported, MPO is an important inflammatory biomarker associated with stroke [51]. Increased serum and plasma levels of MPO have been reported in stroke patients [5]. Low levels of MPO are said to stimulate the various aspects of neurogenesis including cellular differentiation, proliferation, migration and survival of newly formed cells, indicating a close link between neurogenesis and MPO [52]. Kim et al. reported that inhibiting MPO increased the brain-derived neurotrophic factor-cAMP response element binding (BDNF-CREB) signalling, which indicates that increased levels of MPO can adversely affect the signalling pathways involved in neurogenesis [52].

In our study, we observed up regulated MPO expression in the cortical regions up to 72 h post ischemia. Further, we looked into if TG administration after ischemia could ameliorate MPO overexpression or not. We observed MPO down-regulation in the cortical region of the brain administered with TG with a corresponding reduction in the infarct volume (Fig. 7). We also found a concomitant relationship between reduced GSH and MPO expression. As MPO is responsible for generating HOCl at sites of injury and inflammation, which is further associated with a decrease in reduced GSH concentration [53]. Even low concentrations of HOCl can quickly inactivate GPx. H_2O_2 scavenging enzymes like catalase or GPx with GSH can deter HOCl production [54]. Our results are in accordance to the previous study [53]. We believe that TG was able to regulate GSH levels in neurons when administered immediately following an ischemic insult. As mentioned,

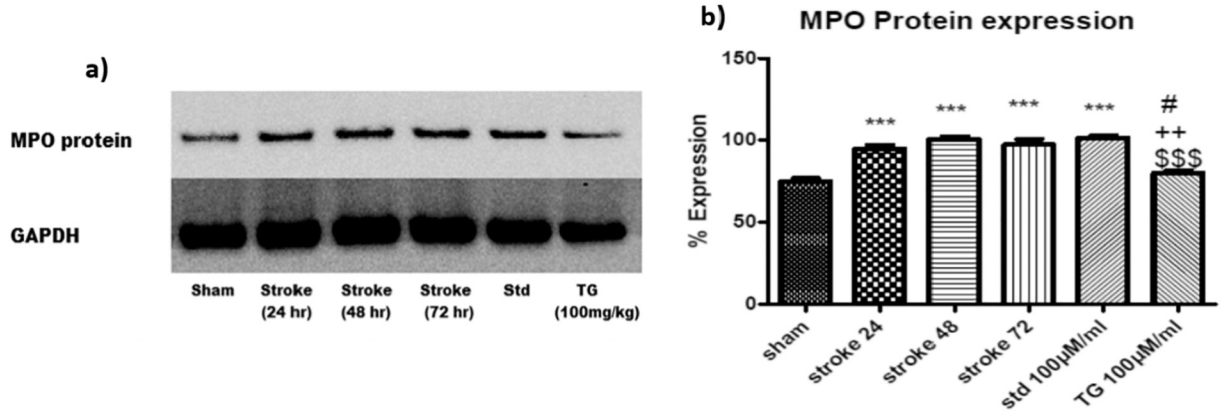

Fig. 7. Western blot analysis of MPO of different set of experimental animals. The data is expressed as mean ± SEM and analyzed for statistical significance using two way ANOVA with Bonferroni post hoc test (***P < 0.001; #P < 0.05; ++P < 0.01; $$$P < 0.001; *vs sham group; #vs stroke 24 h group; $vs stroke 48 h group; +vs stroke 72 h group).

increased levels of GSH could mitigate the detrimental effects seen as a consequence of increased MPO levels following stroke.

In the present study we have observed that TG reduces the expression of MPO and this reduction is taking place in a GSH dependent manner to confer neuroprotection in ischemic stroke. On the basis of our findings, we can infer that TG has neuroprotective properties and was able to render neuroprotection in animal model of ischemic stroke. The future direction of present work includes a pharmacokinetic study and dissecting various mechanisms of neuroprotection where TG may play a promising role in attenuating the progression of ischemic cascade.

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Conflict of interest

The authors have no conflict of interest to declare.

References

[1] U. Dirnagl, Inflammation in stroke: the good, the bad, and the unknown, Neuroinflammation in Stroke, Springer, 2004, pp. 87–99.
[2] S.E. Lakhan, A. Kirchgessner, M. Hofer, Inflammatory mechanisms in ischemic stroke: therapeutic approaches, J. Transl. Med. 7 (2009) 97.
[3] D. Sarmah, H. Kaur, J. Saraf, K. Pravalika, A. Goswami, K. Kalia, et al., Getting closer to an effective intervention of ischemic stroke: the big promise of stem cell, Transl Stroke Res (2017) 1–19.
[4] P. Bhattacharya, A.K. Pandey, S. Paul, R. Patnaik, D.R. Yavagal, Aquaporin-4 inhibition mediates piroxicam-induced neuroprotection against focal cerebral ischemia/reperfusion injury in rodents, PLoS One 8 (2013) e73481.
[5] K. Pravalika, D. Sarmah, H. Kaur, M. Wanve, J. Saraf, K. Kalia, et al., Myeloperoxidase and neurological disorder: a crosstalk, ACS Chem. Neurosci. 9 (2018) 421–430.
[6] D. Sarmah, H. Kaur, J. Saraf, K. Vats, K. Pravalika, M. Wanve, et al., Mitochondrial dysfunction in stroke: implications of stem cell therapy, Transl Stroke Res (2018) 1–16.
[7] N. Lu, Y. Ding, R. Tian, Y.-Y. Peng, Inhibition of myeloperoxidase-mediated oxidative damage by nitrite in SH-SY5Y cells: relevance to neuroprotection in neurodegenerative diseases, Eur. J. Pharmacol. 780 (2016) 142–147.
[8] Y.W. Yap, M. Whiteman, N.S. Cheung, Chlorinative stress: an under appreciated mediator of neurodegeneration? Cell. Signal. 19 (2007) 219–228.
[9] I. Schraufstätter, K. Browne, A. Harris, P.A. Hyslop, J.H. Jackson, O. Quehenberger, et al., Mechanisms of hypochlorite injury of target cells, J. Clin. Investig. 85 (1990) 554.
[10] C.C. Winterbourn, Comparative reactivities of various biological compounds with myeloperoxidase-hydrogen peroxide-chloride, and similarity of oxidant to hypochlorite, Biochim. Biophys. Acta Gen. Subj. 840 (1985) 204–210.
[11] J.M. Pullar, C.C. Winterbourn, M.C. Vissers, Loss of GSH and thiol enzymes in endothelial cells exposed to sublethal concentrations of hypochlorous acid, Am. J. Phys. Heart Circ. Phys. 277 (1999) 1505–1512.
[12] N. Blondeau, The nutraceutical potential of omega-3 alpha-linolenic acid in reducing the consequences of stroke, Biochimie 120 (2016) 49–55.
[13] C. Nguemeni, E. Gouix, M. Bourourou, C. Heurteaux, N. Blondeau, Alpha-linolenic acid: a promising nutraceutical for the prevention of stroke, PharmaNutrition 1 (2013) 1–8.
[14] S. Ilavenil, D.H. Kim, Y.-I. Jeong, M.V. Arasu, M. Vijayakumar, P.N. Prabhu, et al., Trigonelline protects the cardiocyte from hydrogen peroxide induced apoptosis in H9c2 cells, Asian Pac J Trop Med 8 (2015) 263–268.
[15] B. Özçelik, M. Kartal, I. Orhan, Cytotoxicity, antiviral and antimicrobial activities of alkaloids, flavonoids, and phenolic acids, Pharm. Biol. 49 (2011) 396–402.
[16] G.A. Petropoulos, Fenugreek: The Genus *Trigonella*, CRC Press, 2003.
[17] J. Zhou, L. Chan, S. Zhou, Trigonelline: a plant alkaloid with therapeutic potential for diabetes and central nervous system disease, Curr. Med. Chem. 19 (2012) 3523–3531.
[18] J. Folwarczna, A. Janas, M. Pytlik, U. Cegieła, L. Śliwiński, Z. Krivošíková, et al., Effects of trigonelline, an alkaloid present in coffee, on diabetes-induced disorders in the rat skeletal system, Nutrients 8 (2016) 133.
[19] P. Dixit, S. Ghaskadbi, H. Mohan, T.P. Devasagayam, Antioxidant properties of germinated fenugreek seeds, Phytother. Res. 19 (2005) 977–983.
[20] C. Tohda, N. Nakamura, K. Komatsu, M. Hattori, Trigonelline-induced neurite outgrowth in human neuroblastoma SK-N-SH cells, Biol. Pharm. Bull. 22 (1999) 679–682.
[21] A. Vijesh, A.M. Isloor, S. Telkar, T. Arulmoli, H.-K. Fun, Molecular docking studies of some new imidazole derivatives for antimicrobial properties, Arab. J. Chem. 6 (2013) 197–204.
[22] J. van Meerloo, G.J. Kaspers, J. Cloos, Cell sensitivity assays: the MTT assay, Cancer Cell Culture, Springer, 2011, pp. 237–245.
[23] H. Lou, X. Jing, X. Wei, H. Shi, D. Ren, X. Zhang, Naringenin protects against 6-OHDA-induced neurotoxicity via activation of the Nrf2/ARE signaling pathway, Neuropharmacology 79 (2014) 380–388.
[24] P. Klatt, S. Lamas, Regulation of protein function by S-glutathiolation in response to oxidative and nitrosative stress, Eur. J. Biochem. 267 (2000) 4928–4944.
[25] D.A. Dickinson, H.J. Forman, Glutathione in defense and signaling, Ann. N. Y. Acad. Sci. 973 (2002) 488–504.
[26] K.D. Mansfield, M.C. Simon, B. Keith, Hypoxic reduction in cellular glutathione levels requires mitochondrial reactive oxygen species, J. Appl. Physiol. 97 (2004) 1358–1366.
[27] S. Gaweł, M. Wardas, E. Niedworok, P. Wardas, Malondialdehyde (MDA) as a lipid peroxidation marker, Wiad. Lek. 57 (2004) 453–455.
[28] I. Rahman, A. Kode, S.K. Biswas, Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method, Nat. Protoc. 1 (2006) 3159.
[29] D.L. Granger, R.R. Taintor, K.S. Boockvar, J.B. Hibbs Jr., Measurement of nitrate and nitrite in biological samples using nitrate reductase and Griess reaction, Methods in Enzymology, Elsevier, 1996, pp. 142–151.
[30] R. Busto, W.D. Dietrich, M.Y.-T. Globus, I. Valdés, P. Scheinberg, M.D. Ginsberg, Small differences in intraischemic brain temperature critically determine the extent of ischemic neuronal injury, J. Cereb. Blood Flow Metab. 7 (1987) 729–738.
[31] D.R. Yavagal, B. Lin, A.P. Raval, P.S. Garza, C. Dong, W. Zhao, et al., Efficacy and dose-dependent safety of intra-arterial delivery of mesenchymal stem cells in a rodent stroke model, PLoS One 9 (2014) e93735.
[32] L. Belayev, R. Busto, W. Zhao, G. Fernandez, M.D. Ginsberg, Middle cerebral artery

occlusion in the mouse by intraluminal suture coated with poly-L-lysine: neurological and histological validation, Brain Res. 833 (1999) 181–190.
[33] J.J. Ley, A. Vigdorchik, L. Belayev, W. Zhao, R. Busto, L. Khoutorova, et al., Stilbazulenyl nitrone, a second-generation azulenyl nitrone antioxidant, confers enduring neuroprotection in experimental focal cerebral ischemia in the rat: neurobehavior, histopathology, and pharmacokinetics, J. Pharmacol. Exp. Ther. 313 (2005) 1090–1100.
[34] B. Lin, M.D. Ginsberg, Quantitative assessment of the normal cerebral microvasculature by endothelial barrier antigen (EBA) immunohistochemistry: application to focal cerebral ischemia, Brain Res. 865 (2000) 237–244.
[35] E.Z. Longa, P.R. Weinstein, S. Carlson, R. Cummins, Reversible middle cerebral artery occlusion without craniectomy in rats, Stroke 20 (1989) 84–91.
[36] J. Makowska, D. Szczesny, A. Lichucka, A. Giełdoń, L. Chmurzyński, R. Kaliszan, Preliminary studies on trigonelline as potential anti-Alzheimer disease agent: determination by hydrophilic interaction liquid chromatography and modeling of interactions with beta-amyloid, J. Chromatogr. B 968 (2014) 101–104.
[37] J. Nathan, S. Panjwani, V. Mohan, V. Joshi, P.A. Thakurdesai, Efficacy and safety of standardized extract of *Trigonella foenum-graecum* L. seeds as an adjuvant to L-Dopa in the management of patients with Parkinson's disease, Phytother. Res. 28 (2014) 172–178.
[38] S. Zameer, A.K. Najmi, D. Vohora, M. Akhtar, A review on therapeutic potentials of *Trigonella foenum graecum* (fenugreek) and its chemical constituents in neurological disorders: complementary roles to its hypolipidemic, hypoglycemic, and antioxidant potential, Nutr. Neurosci. (2017) 1–7.
[39] A.J. Kettle, C.A. Gedye, C.C. Winterbourn, Mechanism of inactivation of myeloperoxidase by 4-aminobenzoic acid hydrazide, Biochem. J. 321 (1997) 503–508.
[40] E.T. Chouchani, V.R. Pell, A.M. James, L.M. Work, K. Saeb-Parsy, C. Frezza, et al., A unifying mechanism for mitochondrial superoxide production during ischemia-reperfusion injury, Cell Metab. 23 (2016) 254–263.
[41] C. Oliver, P. Starke-Reed, E. Stadtman, G. Liu, J. Carney, R. Floyd, Oxidative damage to brain proteins, loss of glutamine synthetase activity, and production of free radicals during ischemia/reperfusion-induced injury to gerbil brain, Proc. Natl. Acad. Sci. 87 (1990) 5144–5147.
[42] A.A. Al-Majed, F.A. Al-Omar, M.N. Nagi, Neuroprotective effects of thymoquinone against transient forebrain ischemia in the rat hippocampus, Eur. J. Pharmacol. 543 (2006) 40–47.
[43] I.M. Cojocaru, M. Cojocaru, V. Sapira, A. Ionescu, Evaluation of oxidative stress in patients with acute ischemic stroke, Rom. J. Intern. Med. 51 (2013) 97–106.
[44] S. Demirkaya, M. Topcuoglu, A. Aydin, U. Ulas, A. Isimer, O. Vural, Malondialdehyde, glutathione peroxidase and superoxide dismutase in peripheral blood erythrocytes of patients with acute cerebral ischemia, Eur. J. Neurol. 8 (2001) 43–51.
[45] Y. Ergün, M. Üremiş, M. Kılınç, T. Alıcı, Antioxidant effect of Legalon (r) SIL in ischemia-reperfusion injury of rat skeletal muscle, Acta Cir. Bras. 31 (2016) 264–270.
[46] J. Bachevalier, M. Meunier, Cerebral ischemia: are the memory deficits associated with hippocampal cell loss? Hippocampus 6 (1996) 553–560.
[47] F. Block, Global ischemia and behavioural deficits, Prog. Neurobiol. 58 (1999) 279–295.
[48] K. Hattori, H. Lee, P.D. Hurn, B.J. Crain, R.J. Traystman, A.C. DeVries, Cognitive deficits after focal cerebral ischemia in mice, Stroke 31 (2000) 1939–1944.
[49] L.R. Squire, S.M. Zola, Ischemic brain damage and memory impairment: a commentary, Hippocampus 6 (1996) 546–552.
[50] Z. Merali, K. Huang, D. Mikulis, F. Silver, A. Kassner, Evolution of blood-brain-barrier permeability after acute ischemic stroke, PLoS One 12 (2017) e0171558.
[51] V. Dhote, R. Balaraman, Anti-oxidant activity mediated neuroprotective potential of trimetazidine on focal cerebral ischaemia–reperfusion injury in rats, Clin. Exp. Pharmacol. Physiol. 35 (2008) 630–637.
[52] H.J. Kim, P. Leeds, D.M. Chuang, The HDAC inhibitor, sodium butyrate, stimulates neurogenesis in the ischemic brain, J. Neurochem. 110 (2009) 1226–1240.
[53] C.C. Winterbourn, Oxidation of neutrophil glutathione and protein thiols by myeloperoxidase-derived hypochlorous acid, Biochem. J. 327 (1997) 275–281.
[54] O.I. Aruoma, B. Halliwell, Action of hypochlorous acid on the antioxidant protective enzymes superoxide dismutase, catalase and glutathione peroxidase, Biochem. J. 248 (1987) 973–976.